77. The composition of claim 75, wherein said detectable proximity-sensor peptide is a small molecule fluorophore.

Please cancel claims 1-14, and 33.

A marked up version of the foregoing specification amendments is attached hereto.

REMARKS

The October 5, 2001 Official Action and the references cited therein have been carefully considered. In view of the amendments presented herewith and the following remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset, Applicants acknowledge the Examiner's indication that Claim 50 is free of the prior art and is directed to allowable subject matter.

The disclosure has been objected to because of the presence of minor typographical errors. These errors have been corrected in accordance with the present specification amendments.

The disclosure is further objected to for failing to comply with one or more requirements of 37 C.F.R. §1.821 through 1.825. Accordingly, the specification has been amended to comply with the requirements of 37 C.F.R. §1.821 through 1.825. A substitute computer readable copy and paper copy of the Sequence Listing are attached hereto, along with a statement indicating that both the paper copy and C.R.F. are identical and do not introduce new matter into the application.

At page 3 of the Official Action, the Examiner has stated that should claim 13 be found allowable, claim 33 will be objected to under 37 C.F.R. 1.75 as being a substantial duplicate thereof. Accordingly, claim 33 has been canceled, thereby obviating this potential objection.

At page 3 of the Official Action, the Examiner has rejected claims 1-14 under 35 U.S.C. §112, second paragraph as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter regarded as the invention. Claim 1 is allegedly indefinite for reciting the feature "or a fragment thereof". Claim 7 is rejected for recitation of the term "recombinant" because it is not clear if the term refers to the peptide which has been recombined into a different structure or has been prepared by recombinant means. Claim 8 is rejected because there is improper antecedent basis for the recitation of "N-terminal cysteine and C-terminal thioester". Claim 10 is indefinite for including "BODIPY fluorescein" for both the acceptor and donor. Claim 14 is rejected for reciting "a third sensor" which is allegedly vague and indefinite because the location and attachment of the third sensor to the peptide are unclear.

At page 5 of the Official Action, claims 1-4, 6-7, and 9-12 stand rejected under U.S.C. §102(e) as allegedly anticipated by, or in the alternative, under U.S.C. §103 as allegedly obvious over Garman (US 5,011,910) or under U.S.C. §102(b) as allegedly anticipated by the disclosures of each of the following references:

1) Krafft et al. EP No. 0 428 000

2) Marshall and Toth U.S. Patent No.: 5,011,910

3) Meldal and Breddam WO 91/16336

4) Maggiora et al. (1992) J. Med Chem. 35: 3727-3730

5) Geoghegan et al. (1993) Bioconjugate Chem. 4: 537-544

6) Carmel et al. (1973) FEBS Letters 30: 11-14

7) Wang et al. (1990) Tetrahedron Letters 31(45): 6493-6496

8) Ashcom and Jacobson (1989) Analytical Biochemistry 176: 261-264

9) Garcia-Echeverria and Rich (1992) FEBS 297: 100-102

10) Pennington and Thornberry (1994) Peptide Research 7(2): 72-76

11) Matayoshi et al. (1990) Science 247: 954-958

12) Latt et al. (1972) Analytical Chemistry 50(1): 56-62

13) Carmel and Yaron (1978) Eur. J. Biochem. 87: 265-273

14) Yaron et al. (1979) Analytical Biochemistry 95: 228-235

15) Boigegrain et al. (1990) C. R. Acad. Sci. Paris 310, Series III: 465-470

16) Oliveira et al. (1992) Analytical Biochemistry 203: 39-46

17) Miki and Iio (1993) The Journal of Biological Chemistry 268(10): 7101-7106

18) Wang and Liang (1994) Biochemical and Biophysical Research Communications 201(2): 835-840

19) Haugland, (1992) Handbook of Fluorescent Probes and Research Chemicals, 5th Edition: 10-13

20) Tsien et al. WO 92/00388

21) Sparks et al. WO 96/31625

22) Wolfman and Hammes (1977) Biochemistry 16(22): 4806-4811

23) Dobryszycki and Kochman (1988) Biochimica et Biophysica Acta 956: 217-223

According to the Examiner, each of the cited references discloses fluorescence resonance energy transfer peptides or polypeptides having acceptors and donors either at opposite ends or distributed within the sequence. The peptides are used variously as sensors of catalytic activity, conformational changes within the protein, or distance measurements within the peptide or protein.

The foregoing constitutes the entirety of the objection and rejections raised in the October 5, 2001 Office Action.

In accordance with the present amendment, claims 1-14 and 33 have been canceled without prejudice and new claims 51-77 added. Support for the composition claimed in claim 51 can be found in original claims 1 and 16, and at page 6, lines 18-21, wherein it is recited that when practicing the described methods, the conditions of the method will include the composition (substrate), the molecule (enzyme) which acts on the composition to modulate its activity and other reagents or other factors necessary for the activity to occur. Support for the term "modification" is found at page 3, line 18. Support for non-cleavage of the substrate is provided by the aggregate of the examples, wherein none of the described substrates were cleaved in response to exposure to enzyme.

Support for claim 52 is found in original claim 4, at page 3, line 21, and page 23, lines 10-12.

Support for new claims 53 and 54 is also found in original claim 4.

Support for new claim 55 is found in original claim 39 and at page 23, line 16. Support for claim 56 is found in original claim 5. Support for claim 57 is found in original claim 2 and at page 3, lines 14-20. Support for claim 58 is found in original claim 3 and in the specification, for example, at page 3, lines 18-20. Support for claim 59 is also found in original claim 3. Support for the recitation of active enzyme in new claim 60 is found at page 5, line 14 through page 6, line 7. Support for claims 61 and 62 is found in at page 7, lines 7-12. Support for claim 63 is found in original claim 6 and Figure 1. Support for claim 64 is found in original claim 7 and at page 33, lines 15-19. Support for claim 65 is found in original claim 8. Support for claim 66 is found in original claim 9. Support for claim 67 is found

in original claim 10 and at page 8, lines 8-11. Support for claim 68 is found in original claim 11. Support for claim 69 is found in original claim 12. Support for claim 70 is found in original claim 13. Support for claim 71 is found in original claim 14. Support for claim 72 is found at page 7, lines 14-21. Support for claims 73 and 74 is found at page 7, lines 19-21. Support for claim 75 is found in original claim 1 and at page 33, lines 10-12. Support for new claims 76 and 77 is found at page 10, lines 15-24; at page 19, lines 1-4 and at page 24, lines 8-10. Applicants respectfully submit that the newly presented claims are fully supported by the disclosure in the present specification and do not introduce new matter into the application.

NEWLY PRESENTED CLAIMS 51-75 FULLY COMPLY WITH THE REQUIREMENTS OF 35 U.S.C. §112, SECOND PARAGRAPH

The relevant inquiry in determining compliance with the definiteness requirement of 35 U.S.C. §112, second paragraph, is whether the claim in question sets out and circumscribes a particular area with a sufficient degree of precision and particularity, such that the metes and bounds of the claimed invention are reasonably clear. In re Moore, 169 U.S.P.Q. 236 (C.C.P.A. 1971).

The definiteness of claim language may not be analyzed in the abstract, but must be considered in light of the supporting specification, with the language in question being accorded the broadest reasonable interpretation consistent with its ordinary usage in the art. In re Morris, 44 U.S.P.Q. 2d 1023, 1027 (Fed. Cir. 1997). See also Ex parte Cole, 223 U.S.P.Q. 94 (Bd. Apps. 1983) (claims are addressed to the person of average skill in a particular art; compliance with §112 must be adjudged from that perspective, not in a vacuum).

Furthermore, it has long been held that the initial burden of establishing a failure to comply with 35 U.S.C. §112, second paragraph, rests upon the Examiner. In rejecting a claim for alleged indefiniteness, therefore, it is incumbent upon the Examiner to establish that one having ordinary skill in the art would not have been able to ascertain the scope of protection defined by the claim when read in light of the supporting specification. Ex parte Cordova, 10 U.S.P.Q. 2d 1949, 1952 (PTO B.P.A.I. 1988).

When the appropriate procedural approach is followed in assessing the claim terminology at issue herein, it is beyond question that Applicant has satisfied the definiteness requirement of §112, second paragraph, with respect to the subject matter of the amended claims.

Original claim 1 has been canceled, and new claim 51 presented in accordance with the present amendment. The Examiner asserts that recitation of the term "or a fragment thereof" in original claim 1 is vague and indefinite. Claim 51 now recites composition comprising "an enzyme or functional fragment thereof, and a substrate or functional fragment thereof of said enzyzme". One of skill in the art would be readily apprised of the metes and bounds of the claim having Applicants disclosure and claims before them. Applicants respectfully submit that the requirement for functionality removes any ambiguity from the claim. Accordingly, the fragment may be a truncated enzyme or substrate but must retain biological function, e.g., enzymatic activity or the ability to function as a substrate of said enzyme. Support for the use of enzymatically active fragments of the target (substrate) or the enzyme is provided in the specification at page 20, lines 16-22 and page 21, lines 20-21.

To obviate the rejection of claim 7 under 35 U.S.C. §112, new claim 64 is presented which recites that the active enzyme peptide is produced recombinantly thereby removing any

perceived indefiniteness from the claim.

The phrases "N-terminal cysteine and C-terminal "thioester", allegedly lack antecedent basis in claim 8. Claim 8 has been canceled and new claim 65 presented which provides proper antecedent basis for the phrase "said polypeptide further includes an N-terminal cysteine and C-terminal "thioester".

Claim 10 has been rejected under 35 U.S.C. §112, second paragraph, for describing "BODIPY fluorescein" as both the acceptor and donor. Claim 10 has been canceled and new claim 67 drafted in keeping with the Examiner's helpful suggestion to recite "BODIPY FL fluorescein". Support for this amendment is found in the specification at page 4, lines 9-12.

The recitation of "a third sensor" in claim 14 is allegedly vague and indefinite because the location and means of attachment of the third sensor to the peptide are unclear. Applicant strenuously disagrees with the Examiner's position in this regard. As described in the specification and claimed in newly presented claim 71, the positions of the multiple probes in the final construct are selected to report changes in conformation of the polypeptide construct. Thus, the positions may be situated wherever conformational changes are known to occur or are suspected to occur based on the analysis of polypeptides having similar structure/function. One of skill in art would certainly be able to ascertain optimal positions for the incorporation of multiple probes based on the known interactions of, and conformational changes characterized for, the polypeptide molecule or a structurally/functionally homologous polypeptide molecule under different conditions. Support for recitation of a third interacting proximity sensor is provided throughout the specification. See, for example, page 21, line 16 to page 22, line 8 and page 26, lines 9-26.

In light of the foregoing arguments and amendments, Applicant's respectfully submit that metes and bounds of the present claims are clear and unambiguous. Accordingly, Applicant's request that the rejections under 35 U.S.C. §112 second paragraph be withdrawn.

NEWLY PRESENTED CLAIMS 51-77 ARE NOT RENDERED OBVIOUS BY GARMAN

All claim recitations must be considered in determining non-obviousness under 35 U.S.C. §103. In re Sather, 181 U.S.P.Q. 36 (CCPA 1974). It has long been held that when the Examiner disregards specific claim recitations that distinguish over the prior art, the rejection is improper and will be over-turned. In re Glass, 176 U.S.P.Q. 489 (CCPA 1973).

US patent 6,291,201 to Garman et al. describes methods for the preparation of a FRET substrate having donor and acceptor species on opposite sides of a proteolytic cleavage site and wherein the donor and/or acceptor sites are attached to the side chains of the amino acids. Garman teaches that such FRET substrates may be used in assays to identify modulators of protease activity. The FRET substrates described in the '201 patent are cleaved by a protease during the course of the enzymatic reaction. The newly presented claims are directed to the use of FRET labeling and analysis to detect conformational changes in an enzymatic polypeptide or a substrate of the enzymatic polypeptide, wherein the substrate is modified but not proteolytically cleaved as a consequence of the activity of the enzymatic polypeptide.

Garman et al. is silent with regard to the use of FRET pairs to assess conformational alterations that occur as a result of a substrate modification that does not include cleavage of the substrate. It is particularly noteworthy that Garman et al. do not teach or suggest the use of the described

FRET pairs to assess phosphorylation and dephosphorylation of kinase substrates as claimed in claims 53-59 and 72-74. In light of the foregoing, Applicants submit that the Examiner has failed to establish a prima facie case of obviousness based on the subject matter of the newly presented claims. Accordingly, Applicants request that the rejection of the claims under 35 U.S.C. §103 be withdrawn.

NEWLY PRESENTED CLAIMS 51-77 ARE NOT ANTICIPATED BY REFERENCES 1-24 UNDER 35 U.S.C. §102

The law is well settled that a rejection under 35 U.S.C. §102 is proper only when the claimed subject matter is identically disclosed or described in the prior art. In re Arkley, 172 U.S.P.Q. 524 (CCPA 1972). Inasmuch as the numerous references cited in support of the §102 rejections set forth in the October 5 Official Action fail to identically describe the subject matter of Applicant's newly presented claims, these rejections cannot stand.

Because the prior art of record fails to describe or suggest the essential aspects of Applicant's compositions for detecting the effect of an enzyme on a substrate, it cannot reasonably be maintained that these references anticipate the presently claimed invention. Specifically, the prior art fails to disclose compositions comprising active enzymes and substrates wherein an amino acid of the substrate is modified by the enzyme, yet the substrate remains intact.

A number of the cited references, including: Garman (6,291,201); Wang et al. (EP 0 428 000 A1); 5,011,910; Meldal and Breddam (WO 91/16336); Maggiora et al.; Geoghegan et al.; Carmel et al.; Wang et al.; Ashcom and Jacobson; Garcia-Echeverria and Rich; Pennington and Thornberry; Matayoshi et al., Latt et al.; Carmel and Yaron; and Wang and Liang, relate to the use of FRET analysis to detect cleavage of a labeled protease substrate, wherein such cleavage

provides the means to measure protease activity. New claim 51 calls for a composition comprising a polypeptide having enzymatic activity which does not cleave the recited substrate in contrast to the methods and compositions described in each of the references cited above. Cleavage of a FRET-labeled protease substrate results in a change in the FRET emission spectra which registers that the FRET-labels are no longer present on a contiguous or intact substrate, but rather are present on separate, subfragments of the digested substrate. Protease activity, therefore, results in the generation proteolytic subfragments as will be apparent from the following discussion.

As discussed above, in connection with the §103 rejection of claims 1-4, 6, 7, and 9-12, US Patent 6,291,201 to Garman et al. describes methods for the preparation of a FRET substrate having donor and acceptor species on opposite sides of a proteolytic cleavage site wherein the donor and/or acceptor sites are attached via the side chains of amino acids therein. The method further comprises contacting a reactive donor or acceptor species with a polypeptide substrate having the side chains of amino acids therein adapted for reaction with the reactive species and then contacting the substrate so obtained with a corresponding reactive donor or acceptor species. Garman teaches that such methods may be used to prepare novel FRET substrates which may be used in assays to identify modulators of protease activity. This reference is, therefore, directed to the use of FRET labeling and analysis to detect conformational changes in an enzymatic polypeptide or a substrate of the enzymatic polypeptide, wherein the substrate is proteolytically cleaved as a consequence of the activity of the enzymatic polypeptide. Thus, this reference does not anticipate the composition as presently claimed which calls for detecting the effect on an enzyme on a substrate, wherein the enzyme activity produces a modification of an

amino acid substrate, rather than a cleavage of peptide bonds between two substrate amino acids.

Wang et al. (EP 0 428 000 A1) is similar to Garman in that it discusses the use of fluorogenic substrates for the detection of proteolytic enzyme activity. Thus, this reference does not anticipate the composition as presently claimed because it is directed to the use of FRET labeling and analysis to detect conformational changes in an enzymatic polypeptide or a substrate of the enzymatic polypeptide, wherein the enzyme cleaves the substrate.

US Patent 5,011,910 to Marshall et al. is directed to novel fluorogenic substrates for a retroviral protease having a chemical structure X--Thr-Ile--Nle--Phe(Y)--Gln--Arg--NH_2 wherein X is a fluorogenic group and Y is an acceptor for the fluorogenic group, and their use in a fluorometric method for the detection of retroviral protease activity. Thus, this reference does not identically disclose each and every element of the present claims which call for substrates that remain intact in the presence of active enzyme.

Meldal and Breddam (WO 91/16336) describe fluorogenic peptides and their use in the determination of protease cleaving activities. Specifically, the invention relates to peptides which exhibit intramolecular or internal quenching of the fluorescence brought about by a fluorescent chromaphore (donor group) by another chromophore (acceptor group). Table 1 of this application provides data relating to the cleavage of the disclosed peptide substrates. Thus, this reference does not identically disclose the composition of the invention as it teaches cleavage of fluorogenic substrates.

Maggiora et al. describe a general method for the preparation of internally quenched fluorogenic protease substrates using solid-phase peptide synthesis. The kinetic parameters for hydrolysis of synthetic fluorogenic substrates

by renin and HIV proteinase are provided therein. The presently claimed compositions are therefore patentably distinct from those described in Maggiora et al. as they are directed to non-cleavable enzyme substrates.

Geoghegan et al. describe double fluorescent tagging of human renin and collagenase substrate peptides. Such tagged substrate peptides are disclosed as having utility for measuring catalytic cleavage of the peptide substrate by the enzymes disclosed. Thus, this reference also fails to identically disclose the compositions of the present invention.

Carmel at el. provide methods with which to quantitate the activity of hydrolytic enzymes which facilitates analysis of the kinetics of the reaction. The method is based on the interruption of non-radiative energy transfer between two chromophores attached to a substrate molecule. Upon excitation of the donor, enzymatic cleavage is followed by monitoring either an increase in the fluorescence of the donor or a decrease in the fluorescence of the acceptor The compositions of the present claims are, therefore, distinguishable over this reference because the recited substrates are modified, but not cleaved as a consequence of enzymatic activity.

Wang et al. describe the design and synthesis of fluorogenic substrates for HIV protease which are based on resonance energy transfer. These substrates permit sensitive and continuous measurement of HIV protease activity, are cleaved by the protease, and therefore do not anticipate the presently claimed composition.

Ashcom and Jacobson describe the preparation of self-quenched fluorogenic substrates for proteolytic enzymes. Cleavage of such fluorogenic substrates results in an increase in fluorescent emission, which provides means to assay the activity of a proteolytic enzyme. Thus, this reference also

fails to anticipate the presently claimed composition.

Garcia-Echeverria and Rich provide substrates for measuring the catalytic activity of cysteine proteinases. The rate of papain-mediated hydrolysis was monitored by a fluorescence continuous assay based on internal resonance energy transfer in the labeled synthetic peptide substrates. Such substrates were used to evaluate the effect of amino acid substitutions on the kinetic parameters of papain catalyzed hydrolysis. Inasmuch as the methods of this reference calls for cleavage of the fluorogenic substrates, it fails to identically disclose the composition of the present invention.

Pennington and Thornberry describe a peptide substrate for interleukin-1β converting enzyme (ICE) comprised of the protease cleavage site situated between two fluorophores that are located at the termini of the molecule. Upon cleavage of the fluorogenic substrate, an increase in fluorescence is observed, which facilitates a continuous assay of ICE activity. This reference, like the other protease directed references cited by the Examiner, fails to identically disclose the composition as presently claimed.

Matayoshi et al. describe novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. Inasmuch as cleavage of such fluorogenic substrates provides the means to detect and monitor HIV-1 protease activity, Applicant's claimed compositions are patentably distinct.

Latt et al. describe the synthesis and utility of fluorescent substrates for bovine carboxypeptidase A, an enzyme which cleaves peptide bonds adjacent to carboxy terminal aromatic amino acids. For all the reasons described hereinabove, this reference does not anticipate Applicant's claimed compostions.

Carmel and Yaron describe an intramolecularly quenched fluorescent tripeptide substrate of angiotensin-I-

converting enzyme and bacterial dipeptidyl carboxypeptidase. Enzymatic cleavage of the fluorogenic substrate results in a detectable increase in fluorescence emissions. This reference is directed to detection of substrate cleavage as an indicator of enzyme activity and, therefore, fails to anticipate the present invention for the same reasons as described hereinabove.

Yaron et al. provide a review of the literature directed to the design and application of fluorogenic substrates for proteolytic enzymes. The fluorogenic substrates are cleaved by protease enzymes as indicated by an increase in fluorescence. Thus, this reference does not anticipate the compositions presently claimed.

Boigegrain et al. describe fluorogenic peptide substrates for the aspartyl protease pepsin. The proteolytic cleavage of the peptide side chain abolishes the internal energy transfer and is detected by an increase in fluorescence emission, which serves as an indicator of enzymatic activity. As expected, a single amino acid change in a peptide substrate rendered the mutated peptide completely inert to pepsin cleavage. However the reference discloses that this mutated peptide was **not** a substrate for the protease under investigation. Thus, this reference also does not anticipate the compositions of the present invention.

Oliveira et al. describe intramolecularly quenched fluorogenic peptide substrates for human renin. Cleavage of the fluorogenic peptide substrates by renin is detected by an increase in the fluorescence emission of the resultant substrate subfragments. The ability of other hydrolytic enzymes (i.e., pig renin and acidic protease) to cleave the fluorogenic renin substrates was also assessed. Thus, this reference, which also requires cleavage of substrate does not anticipate the composition presently claimed.

Wang and Liang describe fluorogenic substrates which

are comprised entirely of α-amino acids. These substrates were used in continuous assays to measure the enzymatic activity of the protease renin. This reference is silent with regard to the use of enzymes which do not cleave their substrates and thus, is improperly cited under 102(b).

WO 92/00388 of Tsien et al. related to labeled proteins which are described as suitable for determining the presence of cAMP, other second messengers, and organic molecules. A composition of matter is provided wherein **two** labeled proteins are associated in one state and substantially disassociated in another, the equilibrium between which is controlled by the free concentration of an analyte. Inasmuch as the disclosed FRET pair members are placed on two separate proteins in an interacting protein pair rather than on a single molecule as presently claimed, the reference does not anticipate the composition of the invention.

Miki and Iio describe the use of fluorescence resonance energy transfer to examine the kinetics of structural changes of reconstituted skeletal muscle thin filaments. As neither of these molecules comprises an active enzyme, this reference anticipate fails to anticipate the compositions as presently claimed.

WO 96/31625 to Sparks et al. describes polypeptides having functional domains, the nucleic acid sequences encoding them and methods for isolating either the polypeptides or the nucleic acids. This reference does not describe detectable proximity-sensors for incorporation into the polypeptide containing the functional domain or the recognition unit so as to provide means to detect changes in conformation of either polypeptide, which reflect enzymatic activity. Thus, this reference does not identically disclose the composition as presently claimed.

Wolfman and Hammes describe the use of FRET to determine the impact of different effectors on the distance

between two sites in rabbit muscle phosphofructokinase. It is noteworthy that the chemical modifications whereby the rabbit muscle phosphofructokinase was fluorescently labelled rendered the enzyme inactive. See page 4809, first column, first paragraph. In view of the above, this reference does not anticipate the composition as presently claimed which calls for an active enzyme peptide.

Dobryszycki and Kochman describe the use of FRET labeling of amino acid residues of rabbit muscle aldolase to determine changes in the intramolecular distance between the residues that occur in the enzyme upon a change in temperature. It is noteworthy that one of the labels was inserted in the active site of the enzyme which would be expected to render the enzyme inactive. Additionally, this reference fails to disclose a substrate for aldolase. Accordingly, this reference does not anticipate the compositions presently claimed.

The Examiner cites Haugland et al. for disclosure of a variety of FRET pairs. Applicants respectfully submit however, that Haugland et al. fail to make up for the deficiencies of the various references cited under 35 U.S.C. §102.

Given the above-noted patentable distinctions between Applicant's invention and the disclosures of the cited prior art references, the §102 rejections set forth in the October 5, 2001 Official Action cannot be maintained.

In view of the amendments presented herewith and the foregoing remarks, it is respectfully urged that the objections and rejections set forth in the October 5,, 2001 Official Action be withdrawn and that this application be

passed to issue, and such action is earnestly solicited.

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KDR:sjf
Enclosures: Marked up copy showing amendments to specification.

At page 9, line 24 of the specification, please replace the existing paragraph with the following:

--In yet another aspect of the [intention] invention, a method is provided for identifying an agent capable of modulating the activity of a protein kinase capable of phosphorylating Crk-II, by changes in the relative proximity among at least a first position and at least a second position in a modified, [dial] dual-labeled modified Crk-II polypeptide, comprising the steps of:

(a) providing a modified, dual-labeled Crk II molecule such as the Rh-(Crk-II)-Fl construct of Figure 5A and SEQ ID No:8;

(b) subjecting the dual-labeled molecule to conditions inducing the activity in the presence and absence of the agent;

(c) measuring the changes in relative proximity of the first and second interacting proximity-sensor peptides in the composition in the presence and absence of the agent; and

(d) identifying an agent affecting the changes as capable of modulating the activity.--

At page 11, line 16 of the specification, please replace the existing paragraph with the following:

--

Figure 1. Biosensor for c-Abl phosphorylation of the Crk-II adapter protein. c-Abl phosphorylates Crk-II on Tyr 221 which is thought to induce an intramolecular association with the SH2 domain. This rearrangement is expected to yield a net change in the distance between the termini of the protein, which would be reported by a dual-labeled derivative of Crk-II in which the FRET pair tetramethylrhodamine (Rh) and

fluorescein (Fl) are specifically incorporated at its [-] N- and C-termini, respectively.--

At page 19, line 1 of the specification, please replace the existing paragraph with the following:

--An object of the present invention is to provide a generally accessible methodology which allows several recombinant and synthetic polypeptides to be regioselectively linked together, thereby allowing multiple different chemical probes to be site-specifically incorporated into the resulting semi-synthetic protein product. Proteins undergo conformational changes related to their activity or modified state, such as protein targets of phosphorylation and dephosphorylation. By use of probes which are environmentally sensitive, for example, those which are proximity sensitive, changes in their interaction may be monitored to identify the activity or state of the polypeptide. Thus, with a target of biological activity capable of reporting its activity and facile detection of the activity, the target is useful for several purposes. One such purpose is in identifying modulators of the interaction between the target and a molecule which affects the activity or biological state of the target. By way of non-limiting example, which will be exemplified in the Examples below, agents capable of modulating protein kinase activity may be identified using the constructs and methods herein. For example, a protein kinase and its target protein, the latter provided as a semisynthetic construct of the invention labeled to report the state and kinetics of phosphorylation is used. Under appropriate conditions, the combination of the protein kinase and the labeled target reports the protein kinase activity. By carrying out the measurement of the protein kinase activity in this manner in the presence and absence of a candidate agent for modulating

protein kinase activity, one may identify inhibitors or activators of the protein kinase. Moreover, the inhibitors or activators may act on the protein kinase, or on the substrate, or both; further studies may be [preformed] performed to identify the site of interaction. Agents capable of modulating the kinetics of enzymatic activity are detectable using these methods.--

At page 22, line 10 of the specification, please replace the existing paragraph with the following:

--EPL has been extended to permit the insertion of a synthetic peptide into a recombinant protein through the sequential ligation *in solution* of two recombinant protein fragments to the [-] N- and C-termini of a synthetic peptide cassette (17). While this strategy is, theoretically, extendible to the ligation of any combination of synthetic and/or recombinant fragments, the need to perform all of the steps in solution renders the approach technically demanding; after each ligation reaction it is necessary to isolate the desired product from the reaction mixture, a process which is time-consuming and, importantly, leads to substantial handling losses. In principle, these problems should be overcome by transferring the entire process to the solid-phase, in a manner analogous to solid-phase peptide synthesis (SPPS) (18). As with SPPS, this solid-phase protein ligation (SPPL) approach should allow each reaction to be driven to completion by using a large excess of reagents, which can then be simply removed by washing. In addition, there would be no need to isolate intermediate ligation products which would remain immobilized on the support. The present inventors have developed an SPPL technology and have successfully applied it to the generation of a dual-labeled version of the ~35 kDa adapter protein, Crk-II. As is shown herein, this semi-

synthetic protein analog specifically biosenses a post-translational tyrosine phosphorylation event important in regulation of Crk-II mediated signal transduction. Thus, it may be used for various purposes, such as to identify agents capable of modulating phosphorylation activity. It [is] also [only] provides an example for the design of other protein kinase targets, and more broadly, other useful polypeptides [that] wherein biosensing conformational changes [therein is] provides a useful [in] tool for screening and [other purposes] the like, as noted below.

At page 23, line 8 of the specification, please replace the existing paragraph with the following:

--Various polypeptides which undergo conformational changes upon post-translational modification or other effects are candidates for the preparation of a semi-synthetic multiple labeled polypeptide constructs of the invention. Proteins which are themselves targets of enzymatic modification are preferred examples; targets of protein kinase activity are particularly preferred. Non-limiting examples of such targets include transcription factors and signal transduction factors. Numerous other targets are embraced herein, such as those reviewed in (35). In a most preferred embodiment, the polypeptide is an adapter protein. In a more preferred embodiment, the target is a target of the protein kinase c-Abl, such as Crk-II. Figure 1 illustrates the conformational change which the adapter protein undergoes on phosphorylation, and the change in proximity of a dual-labeled composition of the invention comprising the Crk-II polypeptide. The polypeptide of the invention may [be] comprise the sequence of the entire target protein, or may comprise a fragment of the sequence, the fragment which comprises the site of the post-translational modification and the portions of the polypeptide

which undergo the conformational changes to be measured in an aspect of the instant invention. Various modifications which do not detract from the utility of the fragment may be made, for example, to facilitate ligation to the sensor peptides, expression, optimal placement of the sensor peptides, and ease of synthesis or purification, among others.--

At page 24, line 1 of the specification, please replace the existing paragraph with the following:

--Two or more probes may be provided in the semi-synthetic polypeptide. Such probes are selected to report their relative proximities. For example, fluorescence resonance energy transfer (FRET) pairs provide a fluorescence reading depending on the proximity of the fluorophores. For example, fluorescein and tetramethylrhodamine may be used. Other pairs include IAEDANS and fluorescein, EDANS and DABCYL, BODIPY FL fluorescein and BODIPY fluorescein, β-phycoerythrin and CY5, and pyrene and coumarin. FRET pairs are known in the art and a skilled artisan can readily select appropriate pairs for use in the compositions of the invention. The probes of the invention are modified peptides in which the fluorophore or other reporter moiety is provided as a side chain or in the polypeptide backbone. Examples include Dapa-fluorescein (diaminopropionic acid-fluorescein) and N^{α}-tetramethylrhodamine-KRG. Others include peptides or oligopeptides with a moiety, such as EDANS, IAEDANS, DABCYL, BODIPY fluorescein, β-phycoerythrin, CY5, pyrene, or coumarin, capable of participating as a FRET pair with another modified oligopeptide. As noted in the examples herein, which are not limiting, the labeled peptides are provided in forms to be incorporated in a stepwise fashion into the dual-labeled polypeptide. In one synthetic strategy, as described in the Examples below, the labeled peptides may be provided in a form

for eventual enzymatic [of] or chemical cleavage to, for example, release the product from a substrate. Thus, the reactants may have cleavage sites therein to facilitate synthesis. In an example herein, shown in Figure 1, [Crk II] Crk-II (adapter protein; phosphorylation target of c-Abl) is recombinantly expressed as a fusion construct at the N-terminus of an intein-chitin binding domain (Xa-Cys-(Crk-II)-Intein-CBD). An N-terminal cysteine is included to facilitate ligation. The recombinant construct is bound to chitin beads through the chitin-binding domain. In the first step, the above construct is reacted with CGK(Fl)-GLEVFQGPVRKGK(Biotin)GNH_2 ("Cys-Fl-PS-Biotin"; SEQ ID No:6), wherein the N-terminal cysteine is ligated to the Crk-II, forming the product Xa-Cys-(Crk-II)-Fl-PS-Biotin. The ligated product is then bound to avidin beads through the biotin moiety on the C-terminal portion of the fluorescein-labeled peptide. The Xa portion is then cleaved with factor Xa, and the [now-exposed] now exposed N-terminal cysteine reacted with N^{α}-tetramethylrhodamine-KRG-propionamide $^{\alpha}$thioester to ligate the cysteine with the thioester. Subsequently, the PS peptide is cleaved, yielding the dual-labeled product.--

At page 25, line 12 of the specification, pleaes replace the existing paragraph with the following:

--The present invention is directed to the semi-synthetic constructs comprising a target polypeptide and multiple probes, as well as methods for using these constructs in monitoring the biological activity of the polypeptide upon modification (or return to its native state) as well as its use in identifying agents capable of modulating the modification. Numerous examples of polypeptides that are targets of post-translational and other modifications, especially reversible modifications, are available. By way

[on] of non-limiting example, targets of protein kinase activity are preferred embodiments of the present invention. Such include signal transduction factors and transcription factors, as non-limiting examples, as further exemplified in (35). Protein kinases and their phosphorylation/dephosphorylation targets are implicated in critical pathways in which perturbations are known to lead to clinically significant derangements, such as cellular transformation and carcinogenesis. In particular, the protein kinase c-Abl and its target Crk-II are involved in cellular regulation, derangements of which can lead to cellular dysfunctions. Identification of molecules capable of preventing phosphorylation of Crk-II are candidates for pharmaceutical development. Heretofore, assays of compounds for modulation of phosphorylation required the use of ^{32}P and critical measurements of labelling of target molecules. The instant invention provides a facile means to identify modulators of phosphorylation by monitoring changes in the interaction of multiple labels on the phosphorylation target, [induces] induced by changes in [confirmation] conformation consequent to phosphorylation. Rapid, automated high-throughput screening of compounds may be performed using the constructs and methods of the present invention.--

At page 27, line 1 of the specification, please replace the existing paragraph with the following:

--As will be seen in the Examples below, the synthesis was carried out of a semi-synthetic version of the adapter protein, Crk-II, in which the FRET pair, tetramethylrhodamine and fluorescein were incorporated at the [-] N- and C-termini of the protein, respectively (hereafter referred to as Rh-(Crk-II)-Fl), as described in summary above. Crk-II has been implicated in a number of cellular signaling processes, and is

composed predominantly of one Src homology 2 (SH2) and two SH3 domains through which it mediates intermolecular protein-protein interactions (22, 23). Two protein tyrosine kinases, c-Abl and the epidermal growth factor receptor (EGFR), are known to phosphorylate Crk-II on a unique tyrosine residue (Tyr221) located between the SH3 domains (24, 25). This post-translational modification is thought to regulate Crk-II function by inducing an intramolecular association with the SH2 domain (26) which in turn inhibits certain intermolecular protein interactions (22-25). It was anticipated that phosphorylation and subsequent intramolecular association would result in a distance change between the termini of Crk-II, which would lead to a change in FRET between the two fluorophores in the dual-labeled analog (Fig. 1). Consequently, this protein construct would directly biosense this important post-translational event.--

At page 27, line 19 of the specification, please replace the existing paragraph with the following:

--The preparation of the construct Rh-(Crk-II)-Fl is summarized in Fig. 2A. As with SPPS, the strategy can be divided essentially into three parts; attachment of the first building block to a solid support (e.g., avidin beads), chain assembly in a C-to-N direction involving successive deprotection and ligation steps, and cleavage of the completed polypeptide off the solid support. In the first step, full length mouse Crk-II was expressed as an in-frame fusion to an engineered yeast VMA intein which allows the subsequent generation of a reactive $^{\alpha}$thioester derivative of Crk-II. In this example, an extra Gly residue was added to the C-terminus of Crk-II to improve the kinetics of the first ligation reaction (8), and the N-terminal Met was replaced by the sequence -IEGRC (Xa-Cys) (SEQ ID NO:10) to facilitate

controlled sequential ligation (17). Soluble expression of this fusion protein [Xa-Cys-(Crk-II)-Intein-CBD] was optimized using standard protocols (no *in vivo* intein cleavage of the full length fusion could be detected) and the desired material purified by affinity chromatography using a chitin column.--

At page 31, line 1 of the specification, please replace the existing paragraph with the following:

--As will be shown in a further Example, below, phosphorylation studies were performed on the construct to demonstrate its utility in identifying modulators of protein kinase activity. Purified Rh-(Crk-II)-Fl was assayed for its ability to biosense Crk-II phosphorylation by the c-Abl protein tyrosine kinase. As indicated previously, phosphorylation by c-Abl leads to an intramolecular association between a phosphotyrosine motif and the Crk-II SH2 domain, which can be reported by the dual-labeled Crk-II derivative (Fig. 1). Rh-(Crk-II)-Fl was treated with full length recombinant c-Abl and aliquots of the reaction mixture were analyzed by fluorescence spectroscopy and western blotting at ~1 min and 60 min time-points. In the absence of ATP, essentially no change in FRET (i.e. the ratio of the fluorescein/tetramethylrhodamine emission intensities) was observed during the reaction (Fig. 3A), and no Rh-(Crk-II)-Fl phosphorylation could be detected using an anti-phosphotyrosine monoclonal antibody (Fig. 3B). In contrast, when ATP was included in the reaction mixture, a phosphorylation-dependent increase in the emission intensity ratio (a decrease in FRET) was consistently observed. Rh-(Crk-II)-Fl was completely phosphorylated after 1 h as determined by native PAGE mobility (Fig. 3C). The quite modest decrease in FRET (~3% after 60 min) suggests that the SH2-phosphotyrosine interaction, which is triggered by Rh-(Crk-

II)-Fl phosphorylation, results in only a small net change in the relative distance between the [-] N- and C-termini in the protein.--

At page 36, line 18 of the specification, please replace the existing paragraph with the following:

--The resonance energy transfer between the fluorophores in the unphosphorylated molecule was calculated to be 52.5% as determined from both the quenching of the fluorescein emission intensity and the sensitized emission of the rhodamine acceptor (as in ref. 34). Assuming that both fluorophores have random orientations and using a Förster distance of 45 Å for the Fl-Rh pair (34), then the distance between the two fluorophores is ~44 Å. Interestingly, this suggests that unphosphorylated Crk-II has a somewhat compacted domain architecture, as opposed to a linear array of domains; based on the primary sequence, the [-] N- and C-termini could be as much as ~200 Å apart if the inter-domain linkers assume a fully extended conformation.--

At page 42, line 18 of the specification, please replace the existing paragraph with the following:

--In the first step, full length mouse Crk-II was expressed as an in-frame fusion to an engineered yeast VMA intein which allows the subsequent generation of a reactive $^{\alpha}$thioester derivative of Crk-II. An extra Gly residue was added to the C-terminus of Crk-II to improve the kinetics of the first ligation reaction (8), and the N-terminal Met was replaced by the sequence -IEGRC (Xa-Cys) (SEQ ID NO: 10) to facilitate controlled sequential ligation (17). Soluble expression of this fusion protein [Xa-Cys-(Crk-II)-Intein-CBD] was optimized using standard protocols (no *in vivo* intein

cleavage of the full length fusion could be detected) and the desired material purified by affinity chromatography using a chitin column.--

At page 43, line 6 of the specification, please replace the existing paragraph with the following:

--A synthetic peptide, Cys-Fl-PS-Biotin, containing both a fluorescein probe (Fl) and a biotin affinity handle separated by a linker region containing the cleavage site for the PreScission protease [LEVLFQGP, (PS)], (SEQ ID NO: 1) was chemoselectively ligated to the C-terminus of recombinant Crk-II using EPL. This ligation reaction was found to be <95% complete after 48h in the presence of a large excess of peptide and the thiol cofactors ethanethiol and MESNA. Gel filtration was used to separate the unreacted peptide from the desired ligation product which was then attached to monomeric-avidin beads via its biotin functionality. Preliminary model studies had established that the monomeric-avidin-biotin complex was stable to all the washing, deprotection and ligation steps used in SPPL, but that the interaction can be disrupted under mild conditions with exogenous biotin. Trace amounts of unreacted Crk-II protein and any remaining bacterial protein contaminants were then removed by vigorously washing the beads with high salt and detergent at pH 5.2 and pH 8.0. This yielded the pure protein, Xa-Cys-(Crk-II)-Fl-PS-Biotin, immobilized on a solid-support (Fig. 2B, Lane 2).--